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IFN-γ production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8$^+$ T cells

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ABSTRACT

Natural killer T (NKT) cell activation is responsible for eliminating pathogens. However, the biological functions of NKT cells against influenza virus are not fully understood. We therefore investigated the effects of NKT cells in viral infection using CD1d knockout (KO) mice. When CD1d KO or wild-type (WT) mice were infected with a sub-lethal dosage of the influenza virus, the survival rate of CD1d KO mice was significantly lower than for WT mice in association with delayed viral clearance in the lungs. Consistently, IFN-γ production in bronchoalveolar lavage fluid of CD1d KO mice was largely reduced compared to WT mice during infection. Moreover, the cytotoxic activities of NK cells and viral antigen-specific CD8$^+$ T cells were impaired in CD1d KO mice. It was concluded that activated NKT cell-induced IFN-γ release enhances both NK-cell activity and antigen-specific CD8$^+$ T cells to eliminate the influenza virus, thus leading to an enhanced survival.

Introduction

The immune responses for host defenses are mediated by several kinds of cells and cytokines both in innate and in adaptive immunity. Such cells as macrophages, dendritic cells (DCs), natural killer (NK) cells and NKT cells are involved in the innate immune response. NKT cells in mice have been defined as the cells that primarily retain an invariant Vα14-Jα18 rearrangement of the T-cell receptor (TCR) and reactivity to glycolipids presented by the MHC class I-like molecule CD1d (Tupin et al., 2007). NKT cells can produce a large number of cytokines and have surface stimulatory molecules to activate NK cells, T cells, B cells and DCs (Godfrey and Kronenberg, 2004; Gumperz and Brenner, 2001). NKT cells therefore appear to be responsible for the activation of innate immunity and the induction of adaptive immunity. In addition, because those cells can immediately secrete a high amount of IFN-γ after activation and stimulate various immune responses downstream of activated NKT cells, NKT cells therefore play an important role in quickly protecting the host against infectious pathogens, including bacteria, virus, parasite and fungus (Diana and Lehuen, 2009; Tupin et al., 2007; Van Dommelen and Degli-Esposti, 2004).

Because the CD1d molecule is required for the differentiation of Vα14$^+$ NKT cells in the thymus, CD1d KO mice lack Vα14$^+$ NKT cells (Mendiratta et al., 1997; Smiley et al., 1997). These mice have therefore been used for the functional analyses of NKT cells. A previous study demonstrated that CD1d KO mice showed markedly reduced pulmonary eradication of *Pseudomonas aeruginosa* infections and the administration of α-galactosylceramide (α-GalCer), which activates CD1d-restricted NKT cells, rapidly enhanced the pulmonary clearance of the microorganisms along with a large quantity of IFN-γ production by NKT cells (Nieuwenhuis et al., 2002). The roles of NKT cells in antiviral immune responses in mouse have been discussed in several virus infection models using CD1d KO mice. Impaired viral clearance was observed in CD1d KO mice infected with the herpes simplex virus type 1 (Grubor-Bauk et al., 2008, 2003). Moreover, CD1d KO mice showed increased mortality following a high-dose murine cytomegalovirus infection (van Dommelen et al., 2003; Wesley et al., 2008).

A recent mouse study showed that mice administered α-GalCer have enhanced immune protection against influenza virus infection in association with decrease of the virus titer in the lung and an improved weight loss profile (Ho et al., 2008). However, the immunological mechanisms in recovery from influenza virus infection remain unclarified.

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To evaluate NKT cell function after influenza virus infection, CD1d KO mice were used in the present study. The protective roles of NKT cells were then investigated in both the innate and adaptive immune responses against influenza virus infection.

Results

NKT cell-deficient mice exhibit a higher susceptibility to influenza virus infection

Initially, CD1d KO or WT mice were intranasally infected with 10, 100 or 1000 pfu/mouse of influenza virus. When the mice were infected with 10 pfu/mouse of the virus, no mice died in either the CD1d KO or WT groups (Fig. 1A). Conversely, the survival rates of the CD1d KO mice and WT mice infected with 1000 pfu/mouse of the virus were ultimately less than 17% (survival rate; 1/12 in CD1d KO vs. 2/12 in WT mice) by the end of the observation period and no significant differences in the survival rate were observed between the CD1d KO mice and WT mice (Fig. 1C). However, when mice were infected with 100 pfu/mouse of the virus, which is a sub-lethal viral dosage, the survival rate of the CD1d KO mice was significantly reduced compared to the WT mice (Fig. 1B). Moreover, as shown in Fig. 1D, WT mice administered α-GalCer (2 μg/mouse/day × 3 days), which is a specific activator for NKT cells, during influenza viral infection (100 pfu/mouse) markedly protected mice from viral infection in comparison to control mice, thus suggesting that NKT cells play an important role in recovering from the pathogenesis of a sub-lethal dosage of influenza virus.

Clearance of influenza virus and IFN-γ production in impaired lungs in NKT cell-deficient mice

To measure the viral titer and various cytokine levels in the lungs during infection, CD1d KO mice and WT mice were sacrificed on days 2, 4 and 6 following the infection of 100 pfu/mouse of the influenza virus and their BALFs were obtained from each mouse. Fig. 2 shows that the influenza virus titer in the BALF of CD1d KO mice was significantly higher (approximately 10-fold) than in WT mice on days 4 and 6. Correspondingly, in CD1d KO mice, such pro-inflammatory cytokines as TNF-α and MCP-1 in BALF were drastically increased compared to the levels in BALF of WT mice on days 2, 4 and/or 6, respectively (Fig. 3). On the other hand, the production of IFN-γ, which retains its anti-viral effects, was clearly impaired in the BALF of CD1d KO mice. Although the IL-12 p40 production in the CD1d KO mice was comparable to that in the WT mice, the production of IL-12 p70 was below the detection limits in both WT and KO mice during the influenza virus infection (data not shown). These results imply that IFN-γ plays a critical role in the protection of the host from virus-mediated pathogenicity.

There are no differences in cell migration into the lungs between WT mice and CD1d KO mice during influenza virus infection

Analyses of the phenotypes of cells that migrated to the lungs after influenza virus infection (100 pfu/mouse) were performed using XT-2000iV. As shown in Table 1, the numbers of white blood cells (WBC), neutrophils, monocytes, lymphocytes, eosinophils or basophils in the

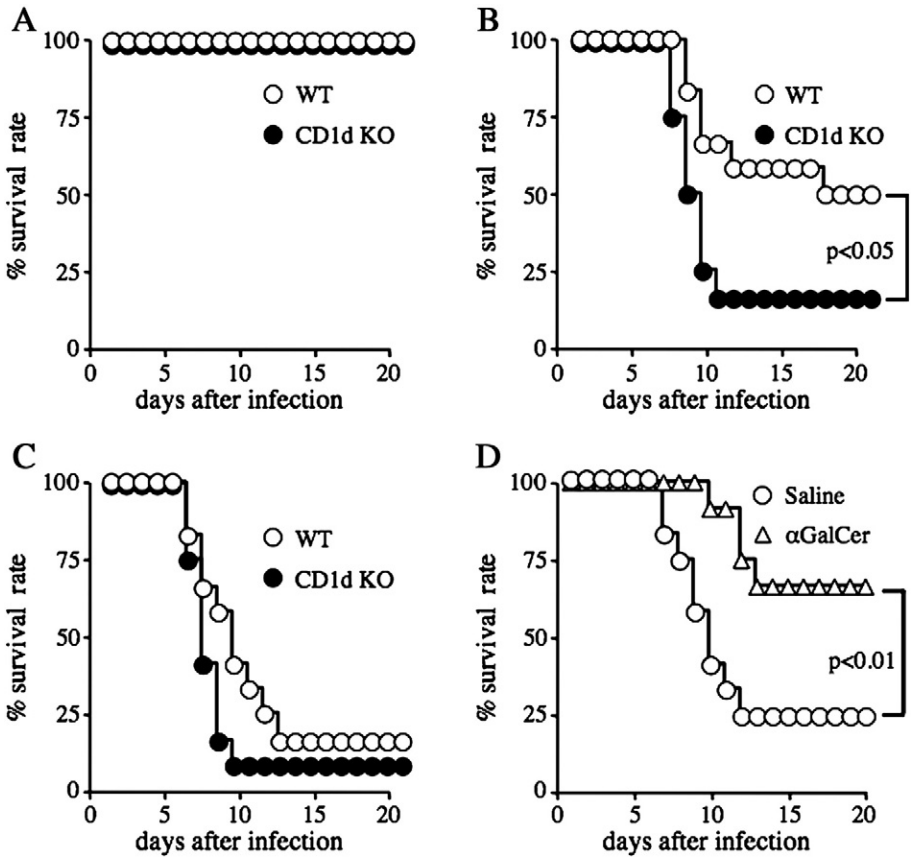

Fig. 1. The survival rate of CD1d KO mice is lower than that of WT mice during influenza virus infection. CD1d KO mice or WT mice were intranasally infected with 10 (A), 100 (B) or 1000 (C) pfu/mouse of influenza virus. (D) WT mice were infected with the influenza virus (100 pfu/mouse) and injected with 2 μg/mouse of αGarCer or saline as a control on days 0, 3 and 5. The survival rate was monitored daily until day 20. Twelve mice were evaluated for each group. Similar results were obtained in two independent experiments.

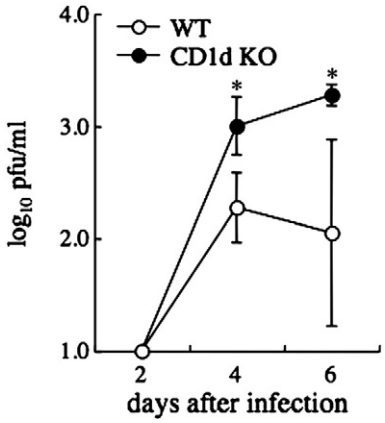

Fig. 2. Delay of the viral clearance in the lungs of CD1d KO mice during influenza virus infection. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus. On days 2, 4 and 6 after the infection, mice were sacrificed and the BALF supernatant was prepared to measure virus titer. The virus titer was determined by a plaque assay as described in Materials and methods. Six mice were used in each group. $^{*}p<0.05$ compared to the viral titer of WT mice.

BALF of WT mice were comparable to those of CD1d KO mice during influenza virus infection.

The enhancement of IFN-γ downstream of activated NKT cells is responsible for an improved survival rate after influenza virus infection

Impaired IFN-γ production was observed in CD1d KO mice during influenza virus infection (Fig. 3). To confirm whether IFN-γ production contributes to improved survival rates following infection, WT mice were treated with a neutralizing anti-IFN-γ Ab or a normal rabbit IgG_1 Ab on days 0 and 5 of infection and the survival rates were monitored until day 20. As a result, infected WT mice treated with the anti-IFN-γ Ab exhibited a lower survival rate in comparison to control mice (Fig. 4). However, the survival rate of CD1d KO mice given an anti-IFN-γ Ab was comparable to that of CD1d KO mice treated with the isotype control (IC) antibody (survival rate on day 20; 1/12 in anti-IFN-γ Ab treated group vs. 2/12 in IC group). In addition, the survival of infected mice treated with IC (Fig. 4) was not significantly different from that of infected WT mice (Fig. 1B) ($p=0.3954$). These results demonstrated that IFN-γ produced by activated NKT cells mediates anti-viral immunity downstream of NKT cells.

Table 1
Changes of cellular composition in the BALF after influenza virus infection ($\times 10^5$ cell).

		Before	Day 2	Day 4	Day 6
WBC	WT	5±0.5	7±2.6	92±8.2	99±18.7
	CD1d K.O.	7±4.2	6±1.6	107±12.1	80±9.4
Neutrophil	WT	1±0.6	2±0.9	16±2.6	12±3.2
	CD1d K.O.	1±1.2	1±0.8	16±2.0	12±1.3
Monocyte	WT	3±0.5	4±1.6	70±4.6	74±8.2
	CD1d K.O.	5±3.0	4±1.0	85±11.0	64±8.7
Lymphocyte	WT	$<10^5$	$<10^5$	4±0.6	5±1.3
	CD1d K.O.	$<10^5$	$<10^5$	5±1.5	4±0.9
Eosiophil	WT	$<10^5$	$<10^5$	1±0.3	1±0.2
	CD1d K.O.	$<10^5$	$<10^5$	1±0.5	1±0.2
Basophil	WT	$<10^5$	$<10^5$	$<10^5$	$<10^5$
	CD1d K.O.	$<10^5$	$<10^5$	$<10^5$	$<10^5$

The mean and standard deviation from 3 mice/group is shown.

IFN-γ production enhanced by activated NKT cells enhances the cytotoxic activity of NK cells

It is well known that IFN-γ enhances the activity of various cells, thereby mediating cellular immunity. To explore the mechanisms of IFN-γ production by NKT cells and mediation of the immune responses against the influenza virus, NK activity was examined. The frequency and number of NK cells in the BALF of CD1d KO mice were comparable to that of WT mice (Fig. 5A and B). The cytotoxicity of NK cells in BALF on day 6 of infection was examined with a co-culture system using effector/target (E/T) cell ratios of 30:1, 3:1 and 1:1. The cytotoxicity of NK cells in an E/T ratio of 30:1 was significantly reduced in CD1d KO mice in comparison to WT mice, although the cytotoxicity of NK cells in an E/T ratio of 3:1 and 1:1 were close to the limit of detection in both groups (Fig. 5C).

IFN-γ production downstream of activated NKT cells also increases $CD8^+$ T cell killing activity

The effects of IFN-γ produced by NKT cells during viral infection on the number and the function of viral antigen-specific $CD8^+$ T cells were next examined. There was no difference in the frequency of $CD8^+$ T cells in the BALF of CD1d KO mice or WT mice (Fig. 6A). However, the number of $CD8^+$ T cells was significantly reduced in CD1d KO mice comparing to WT mice on day 6 of infection (Fig. 6B). Next, the killing activity of antigen-specific $CD8^+$ T cell in the lung was determined using an *in vivo* killing assay. CD1d KO mice or WT mice were transferred with an equal mixture of peptide-pulsed $CFSE^{low}$ cells and unpulsed $CFSE^{high}$ cells on day 6 of infection as described in Materials

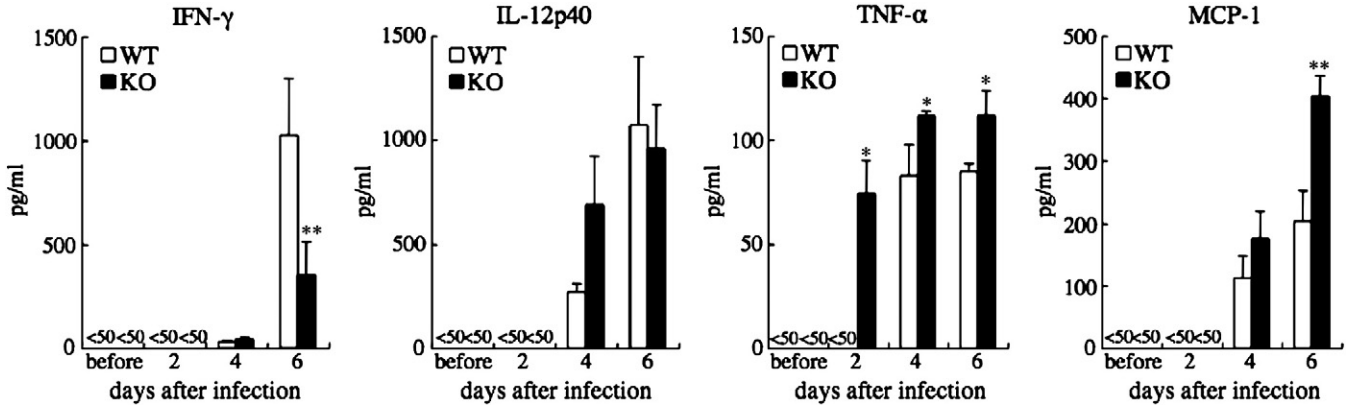

Fig. 3. Differences in cytokine production between CD1d KO mice and WT mice infected with the influenza virus. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus. BALFs were obtained on days 2, 4 and 6 and IFN-γ, IL-12, TNF-α and MCP-1 were measured by ELISA in the supernatants. Six mice were used in each group. $^{*}p<0.05$, $^{**}p<0.01$ compared to cytokine productions of WT mice.

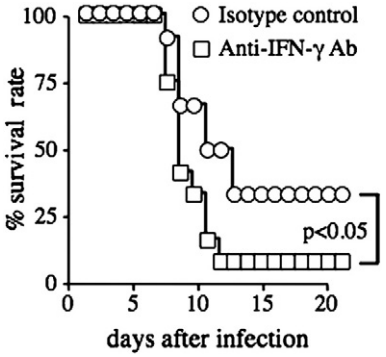

Fig. 4. Neutralization of IFN-γ increases the susceptibility to influenza virus infection in WT mice. WT mice intranasally infected with 100 pfu/mouse were intravenously administered a neutralizing anti-IFN-γ Ab or a normal rabbit IgG_1 as an isotype control on days 0 and 5. The survival rate of the mice was monitored daily until day 20. Twelve mice were used in each group.

and methods. As shown in Fig. 6C, a similar *in vivo* killing intensity was observed in both CD1d KO mice and in WT mice infected with the influenza virus, but was not observed in the non-infected mice. When the percentage of killing activity was calculated, the activity in CD1d KO mice infected with the virus was significantly reduced in comparison to the infected WT mice (Fig. 6D).

Neutralization of IFN-γ abrogates virus clearance with impaired NK cell and CD8 T cell cytotoxicity

Neutralization of IFN-γ significantly decreased the survival of mice during influenza virus infection (Fig. 4). Following this observation, we investigated whether the neutralization of IFN-γ has an effect on the virus clearance, cytotoxicity of NK cells or of viral antigen-specific $CD8^+$ T cells in the BALF on day 6 of the virus infection. The clearance of influenza virus was significantly delayed in IFN-γ neutralized mice compared to control mice (Fig. 7A). In accordance with the delayed virus clearance, the cytotoxicity of NK cell tended to be lower in mice treated with the anti-IFN-γ Ab than mice treated with IC (Fig. 7B). Of note, the viral antigen-specific cytotoxicity of $CD8^+$ T cells in IFN-γ neutralized mice was significantly abrogated compared to that in control mice, and the cytotoxicity of CD8 T cells was abolished almost completely by IFN-γ neutralization.

Asialo-GM1 positive and CD8 positive cells are responsible for IFN-γ production during influenza virus infection

Because IFN-γ production downstream of NKT cell activation plays a crucial role in protecting animals from influenza virus infection, candidates cells responsible for the production of IFN-γ activated by NKT cells were investigated *in vivo* using antibody depletion on day 6 of the infection. As shown in Fig. 8, IFN-γ production in WT mice was significantly higher than that in CD1d KO mice (as was also indicated in Fig. 3). When either the anti-CD8 Ab or anti-asialo GM1 Ab used to deplete the cells expressing the molecules were administrated to infected WT mice, the IFN-γ in the BALF decreased to a level comparable to infected CD1d KO mice. However, the administration of an anti-CD4 Ab did not lead to this effect. These results suggest that the major source of IFN-γ on day 6 after infection was the asialo-GM1 positive and CD8 positive cells. It is therefore likely that activated NKT cells stimulate $CD8^+$/asialo-$GM1^+$ cells, thus resulting in an enhanced IFN-γ production in the cells.

Discussion

Studies using such knockout mice as CD1d or Jα18 and studies using αGalCer have been mainly used to investigate the roles of NKT cells in an infectious mouse model (Duthie et al., 2005; Exley et al., 2001; Gonzalez-Aseguinolaza et al., 2000; Johnson et al., 2002; Kakimi et al., 2000). While accumulating reports suggest that NKT cells play a pivotal role in eliminating infective microorganisms including bacteria, virus, parasite and fungus, the effect of NKT cells on immune responses against influenza virus infection has yet to be elucidated (De Santo et al., 2008; Ho et al., 2008; Tupin et al., 2007; Van Dommelen and Degli-Esposti, 2004). In the present study, it has been demonstrated that IFN-γ induced by activated NKT cells in mice infected with the influenza virus enhances the cytolytic activities both of NK cells and viral antigen-specific $CD8^+$ T cells to eliminate the virus. Various kinds of immune cells produce IFN-γ. Although NKT cells are the primary members for innate immunity, IFN-γ involving in activated NKT cells is essential for innate immunity through NK cell activation and in adaptive immunity through Ag-specific CTL activation as presented in this study.

It has been reported that one immune modulatory function of IFN-γ produced by activated NKT cells is to rapidly enhance both the cytotoxicity and IFN-γ production of NK cells for host innate immunity (Carnaud et al., 1999). Our results provide evidence that CD1d KO mice with impaired IFN-γ production or neutralization of IFN-γ in infected WT mice exhibit incomplete cytotoxicity of NK cells after influenza virus

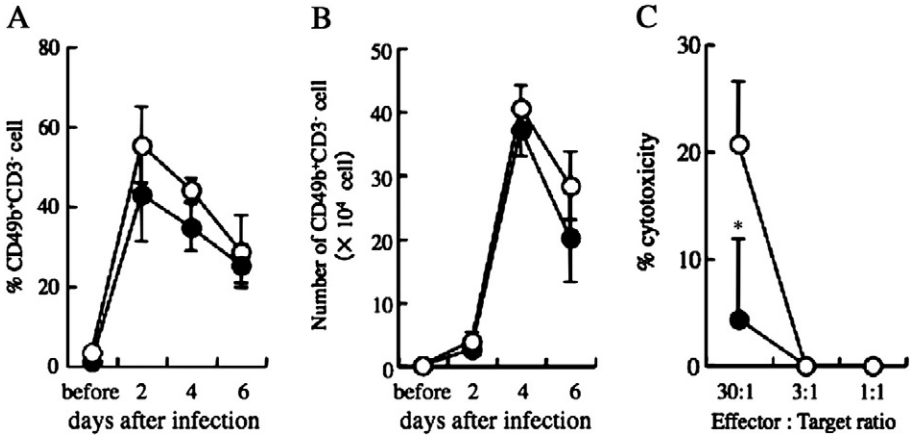

Fig. 5. Migration to the lung and cytotoxic activity of NK cells after influenza virus infection. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus. (A) Cells in the BALFs were analyzed by flow cytometry and CD49b-positive and CD3-negative lymphocytes were identified to be NK cells. (B) The number of NK cells in the BALFs was calculated. (C) The cells in the BALFs were considered to be effector cells and were prepared from CD1d KO mice or WT mice on day 6 following infection. The titrated effector cells and YAC-1 cells were mixed at various effector/target (E/T) ratios as indicated. The cytotoxicity percentages were calculated as described in Materials and methods. *$p<0.05$ compared to the viral titer of WT mice.

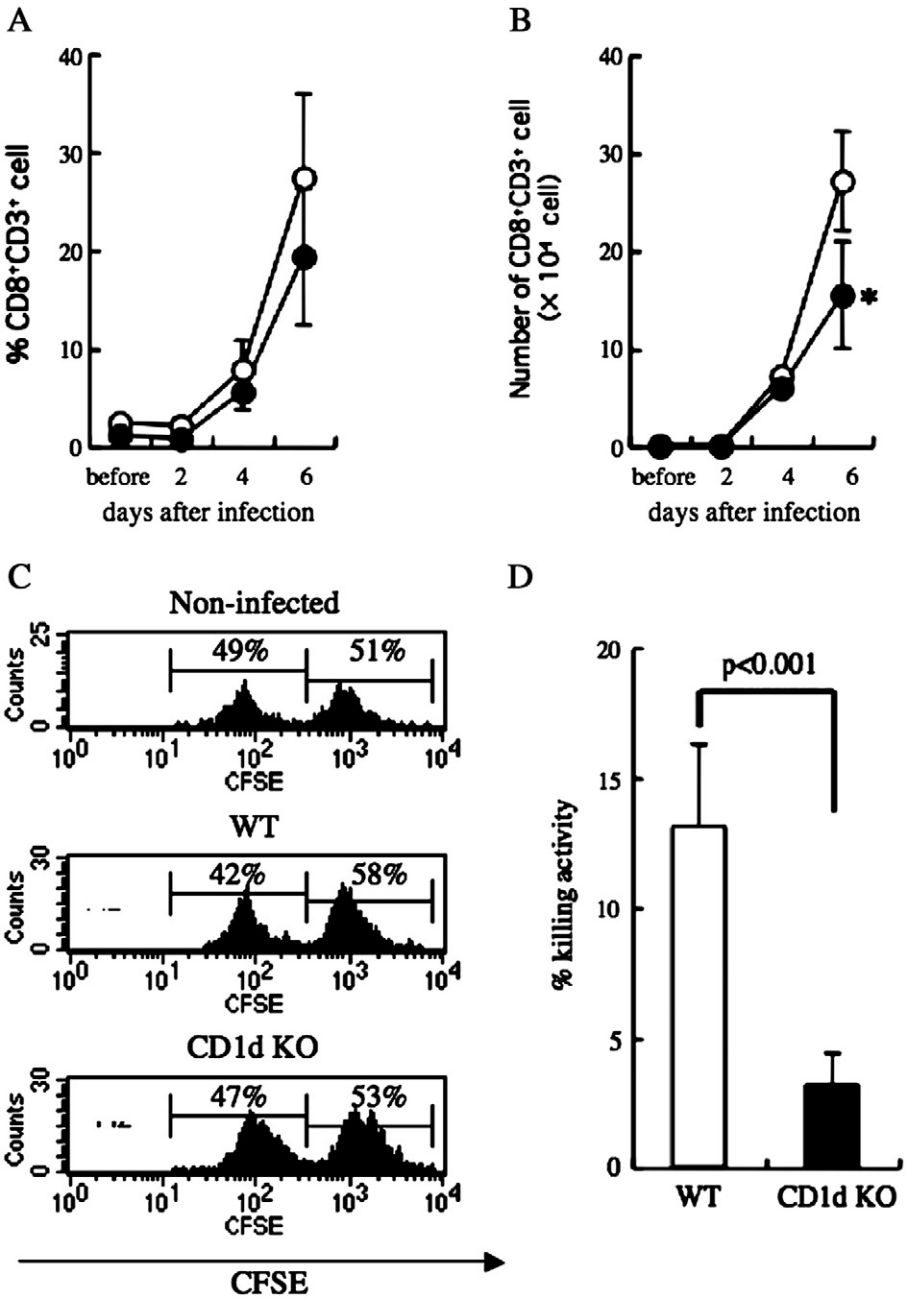

Fig. 6. Migration of $CD8^+$ T cells to the lung and the antigen-specific cytotoxicity of $CD8^+$ T cells following influenza virus infection. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus. (A) The cells in the BALFs were stained with a PE-anti-CD8α Ab and a FITC-anti-CD3ε Ab and were analyzed by flow cytometry. CD8-positive and CD3-positive lymphocytes were identified to be $CD8^+$ T cells. (B) The number of $CD8^+$ T cells in the BALFs was calculated. (C) Ten million cells, equally composed of $NP_{147\text{-}155}$ pulsed $CFSE^{low}$ cells and unpulsed $CFSE^{high}$ cells, were injected i.v. into non-infected mice, infected CD1d KO mice or infected WT mice on day 6 following influenza virus infection. Four hours later, mice were sacrificed and the lungs removed prior to the FCM analyses. The representative data of six individual mice from non-infected mice, infected WT mice or infected CD1d KO mice are shown. (D) Percentage of in vivo killing activity for infected CD1d KO mice or infected WT mice were calculated as indicated in Materials and methods.

infection. Because NK cells have the potential to effectively eliminate the infected cells due to detection of the lack of MHC class I expression, the depletion of NK cells increases the susceptibility to influenza virus infection, therefore leading to higher morbidity and mortality rates (Stein-Streilein and Guffee, 1986). Moreover, it has recently been shown that the natural cytotoxicity receptors NKp46 and NKp44 on human NK cells recognize influenza virus hemagglutinins (HA) on virally infected target cells (Arnon et al., 2001; Mandelboim et al., 2001). Subsequently, the natural cytotoxicity receptor 1 (NCR1) on NK cells in mice was identified to be a mouse homolog of human NKp46 and lacking NCR1 resulting in increased morbidity and mortality in vivo with insufficient eradication of the influenza virus (Gazit et al., 2006). These reports suggest that NK cell activation is responsible for the primary host defense against influenza virus infection in innate immunity.

The ability of virus-specific cytotoxic $CD8^+$ T cells to thoroughly prevent the influenza virus from escaping from the innate immune response has been demonstrated in $CD8^+$ T cell-deficient mice (Bender et al., 1992). Although the number of viral antigen-specific $CD8^+$ T cells in the lungs during influenza virus infection was not determined in present study, some reports have clearly demonstrated that viral antigen-specific $CD8^+$ T cells infiltrate the lungs from around day 3 following infection and that their migration is enhanced by α-GalCer treatment (Ho et al., 2008). Moreover, both invariant NKT cells and CD1d molecule-dependent $CD8^+$ T cell migration to the lungs have

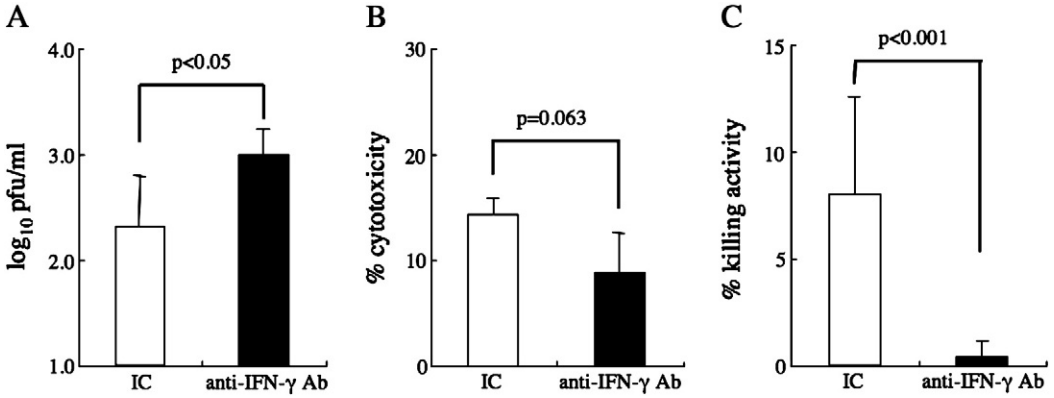

Fig. 7. Effects of neutralization of IFN-γ on virus clearance, cytotoxicity of NK cells, and antigen-specific $CD8^+$ T cells following influenza virus infection. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus and 25 μg/mouse of anti-IFN-γ Ab or an equivalent amount of IC were administrated i.v. on days 0 and 5. (A) The virus titer in the BALF was measured by plaque assay on day 6 after the virus infection. Six mice were used in each group. (B) NK cell cytotoxicity in the BALF was determined on day 6 following infection as described in Materials and methods. The effector cells and YAC-1 cells were mixed at a 30:1 (E/T) ratio. (C) The cytotoxicity of virus-specific $CD8^+$ T cells in the lungs was determined on day 6 following infection, as described in Materials and methods. Four mice were used in each group.

been shown (De Santo et al., 2008). The influenza viral nucleoprotein (NP) peptide was used to measure the antigen-specific killing activity of $CD8^+$ T cells in the present study, as demonstrated by Wysocka and Bennink (1988), the authors clearly showed that the cytotoxic $CD8^+$ T cell responses against influenza virus infection in BALB/c ($H\text{-}2K^d$) mice are dominated by clones reactive to the viral NP, in which approximately 75% of cytotoxic $CD8^+$ T cells are NP-reactive clones and the other viral proteins share the remains. Our findings indicate that defective IFN-γ production in CD1d KO mice or neutralization of IFN-γ in infected WT mice lead to impaired activation of cytotoxic $CD8^+$ T cells in vivo and may be associated with a higher mortality and delayed clearance of the virus escaping from the innate immunity. Stober et al. (2003) have reported that NKT cells contribute to DC-dependent priming of MHC-class I restricted $CD8^+$ T cells. However, the direct effect of DCs on priming Ag-specific CTL could not be shown in this study, but we hypothesize that the present results are consistent with those findings.

The major IFN-γ producing cells in mice infected with influenza virus were also identified by this study. We showed that both CD8 positive and asialo-GM1 positive cells are the major IFN-γ producing cells on day 6 after infection, although the phenotypic features and

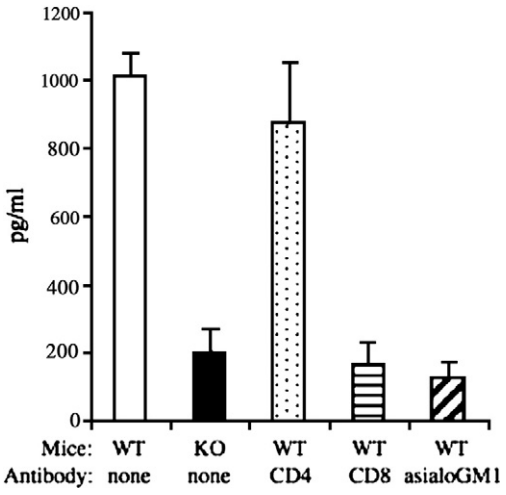

Fig. 8. The influence of IFN-γ production on influenza virus infection following antibody-mediated cell depletion. CD1d KO mice or WT mice were intranasally infected with 100 pfu/mouse of the influenza virus and 25 μg/mouse of anti-CD4 Ab or anti-CD4 Ab or 50 μ of anti-asialo GM1 serum, respectively, were i.v. injected on days −3, 0 and 3. Mice were sacrificed on day 6 after the virus infection and IFN-γ in the BALF was measured by ELISA assay. Five mice were used for each group.

kinetics of the cells have not yet been precisely identified. It has been reported that $CD8^+$/asialo-$GM1^+$ cells, which comprise only 3% of the naïve BALB/c $CD8^+$ T cells in splenocytes, are major producers of IFN-γ among the $CD8^+$ cell population upon stimulation with anti-CD3 (Kosaka et al., 2007). It is plausible that $CD8^+$/asialo-$GM1^+$ cells might be a subpopulation of NKT cells. However, $CD8^+$ NKT cells have only been reported in human lymphocytes (Kulkarni et al.,), so $CD8^+$ NKT cells can be ruled out as the major IFN-γ producing cells in this study.

There are two activation pathways from DCs to NKT cells; direct activation and indirect activation (Tupin et al., 2007). In some types of bacterial infections, NKT cells can directly recognize several bacterial glycolipid antigens. For example, phosphatidylinositol tetramannoside (PIM4) and monogalactosyl diacylglycerol lipids are recognized in *Mycobacteria* spp. and *Borrelia burgdoferi* infection, respectively (Kinjo et al., 2006, 2005; Mattner et al., 2005). On the other hand, it has not yet been reported that NKT cells recognize lipid antigens derived from viral particles (Cerundolo et al., 2009). It appears that indirect activation of NKT cells is the primary pathway in viral infections. In indirect NKT cell activation following viral infection, there have been reported two pathways, the endogenous antigen- and cytokine-mediated pathways (Tupin et al., 2007). In the endogenous antigen-mediated pathway, NKT cells can be promptly activated by inflammatory cytokines from antigen-presenting cells that recognize microorganisms in combination with CD1d-mediated presentation of endogenous ligands derived from viral infection. In the cytokine-mediated pathway, viral TLR ligands are recognized by the TLRs of DCs, and cytokines such as IL-12 and IL-18 are released from the DCs and are sufficient for IFN-γ production of NKT cells (Brigl et al., 2003; Nagarajan and Kronenberg, 2007; Van Dommelen and Degli-Esposti, 2004). However, because antigen presentations via CD1d are not required in the cytokine pathway, this pathway is likely not involved in the influenza virus infection model of the present study.

Recently, De Santo et al. (2008) demonstrated that invariant NKT cells regulate the migration and function of myeloid-derived suppressor cells (MDSCs) during influenza virus infection. They also demonstrated that one of the mechanisms responsible for controlling MDSCs is the suppressive function by invariant NKT cells through CD1d and CD40 molecules on MDSCs. Therefore, lung MDSCs induced by influenza virus infection suppress the expansion and function of viral antigen-specific $CD8^+$ T cell immune responses in the absence of invariant NKT cells resulting in a high virus titer and increased mortality. Impaired virus clearance and decreased function of viral antigen-specific $CD8^+$ T cells was found to be associated with CD1d

KO mice during influenza virus infection in the present study, suggesting that MDSCs may be involved in the impaired $CD8^+$ T cell functions in infected CD1d KO mice.

In conclusion, the current study provides evidence that NKT cells are important in inducing fully functional innate and adaptive immune responses to eliminate the influenza virus from an infected host. Our data also suggest that this protective mechanism may be dependent on sufficient IFN-γ downstream of the activated NKT cells activated by cytokines derived from DCs that recognize microorganisms. Future research will examine the efficient control of innate and adaptive immune responses against the HINI swine flu and H5N1 avian influenza virus by utilizing αGalCer administration.

Materials and methods

Mice

BALB/c female WT mice (6 weeks of age) were purchased from the Oriental Yeast Co., LTD. (Tokyo, Japan). CD1d KO mice, bred on a BALB/c background, were bred in our experimental animal facility. All mice were housed under specific pathogen-free conditions according to the animal protocol guidelines of the Committee on Animal Care of Tokyo Medical University (protocol No. S-47).

Influenza virus infection and treatment protocols

Influenza virus A/Puerto Rico/8/34 (PR8, H1N1) strain was kindly provided by Dr. Kawaoka at the University of Tokyo. Mice were anesthetized and intranasally infected with 20 μl of saline containing the virus. Day 0 was defined as the day of viral infection throughout the experiments and the survival was monitored daily until 20 days after the infection. In some experiments, mice were administered by intraperitoneally (i.p.) administered 2 μg/mouse of αGalCer (Kirin Holding Company, LTD. Tokyo, Japan) on days 0, 3 and 5. For the neutralization of IFN-γ, 25 μg/mouse of anti-IFN-γ Ab (clone: XMG1.2) (BD Pharmingen, CA, USA) or an equivalent amount of normal rabbit IgG_1 as a control (clone: R3-34) (BD Pharmingen) were intravenously (i.v.) injected on days 0 and 5. To deplete specific cell types, the mice were injected intravenously with 25 μg/mouse of anti-CD4 Ab (clone: H129.19), anti-CD8 Ab (clone: 53-6.7) or 50 μl/mouse of anti-asialo GM1 Ab on days -3, 0 and 3, respectively.

Plaque assay

CD1d KO mice and WT mice were in intranasally infected with 100 pfu/mouse of influenza virus and the virus titers in bronchoalveolar lavage fluid (BALF) were determined by a plaque titration. Confluent of Madin Darby canine kidney (MDCK) cells in 12 well plates were washed three times with serum-free DMEM and subsequently with DMEM supplemented with 0.5% BSA, 100 μg ml^{-1} DEAE dextran, 1 μg ml^{-1} TPCK-treated trypsin, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma, MO, USA) were added in each well followed by incubation with serially diluted BALF supernatants at 33 °C for 1 h. After gently washing, the cells were overlaid with the medium containing 0.9% agar and were maintained in 5% CO_2 at 33 °C for 3 days. The agar media were then removed and the cells were fixed with 5% formalin solution and stained with a 0.1% crystal violet solution.

Cytokine assay in BALF

The concentration of cytokines in the BALF supernatants were measured using a mouse IFN-γ ELISA development kits, mouse IL-12 p40 ELISA development kits, mouse IL-12 p70 ELISA development kits, mouse TNF-α ELISA development kits and a mouse CCL2/JE/MCP-1 immunoassay kit (R&D systems, MN, USA) according to the manufacturer's instructions as described previously (Ishikawa et al., 2008).

Analysis of cell migration into the lungs following virus infection

To evaluate the number of cells migrating the lung after influenza virus infection, the cells in the BALF obtained from uninfected or infected mice were washed with PBS, and their number and type of cells were analyzed by XT-2000iV, which is an automatic blood cell analysis apparatus (Sysmex corporation, Hyogo, Japan).

Flow cytometric analysis

The flow cytometric (FCM) analyses for cells in BALF were performed on a FACSCalibur flow cytometer with the Cellquest Pro software program as previously described (Ishikawa et al., 2007). The cells were stained in PBS with 1% BSA and then fixed with 1% paraformaldehyde. The antibodies used in the present study were anti-CD16/CD32 Ab (clone: 2.4G2) as an Fc receptor-blocker, FITC-conjugated anti-CD3ε Ab (clone: 145-2C11), FITC-conjugated anti-CD49b Ab (clone: DX5), PE-conjugated anti-CD3ε Ab (clone: 145-2C11) and PE-conjugated anti-CD8α Ab (clone: 53-6.7) (BD Pharmingen).

Cytotoxic activity assay for NK cells

The cytotoxicity of NK cell in BALFs were examined by a co-culture system with YAC-1 cells as the target cell (Noda et al., 2001) and the levels of lactate dehydrogenase (LDH) in the culture supernatants, which is an enzyme released upon cell lysis, were measured using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, WI, USA). To prepare the effector cells, cells in BALFs were obtained from CD1d KO mice or WT mice on day 6 of infection. Serially titrated effector cells were co-cultured with 1×10^5 cell/ml of target cells (YAC-1) in DMEM supplemented with 10% FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a 96-well-round bottom microplate at 37 °C for 4 h. The level of LDH in the culture supernatants was then measured and the NK cell cytotoxicity was calculated according to the manufacturer's protocol.

In vivo killing assay

An in vivo killing assay for antigen-specific $CD8^+$ T cells was performed as described (Barber et al., 2003), and single splenocytes from naïve mice were stained with a PKH26 red fluorescent cell linker kit for general cell membrane labeling (Sigma). The cells were separated into two equal populations prior to labeling with 50 nM CFSE (Sigma) (defined as $CFSE^{low}$) or 500 nM CFSE (defined as $CFSE^{high}$). The $CFSE^{low}$ cells were pulsed with 10 μM of nucleoprotein (NP) $_{147\text{-}155}$ of the influenza virus PR8 strain, which is the TYQRTRALV peptides sequence-restricted MHC class I ($H\text{-}2K^d$), for 30 min, while $CFSE^{high}$ cells were not pulsed. After washing, the two populations were mixed in equal proportions and 1×10^7 cells were injected i.v. into the non-infected control mice or mice infected with influenza virus on day 6. At 4 h after the injection, lungs were removed from the mice and single cells were analyzed by an FCM to measure the levels of cytotoxicity. The percent of killing activity was calculated by the following formula: Percent of killing activity = 100 − {[(% $CFSE^{low}$ in infected mice / % $CFSE^{high}$ in infected mice) / (% $CFSE^{low}$ in uninfected mice / % $CFSE^{high}$ in uninfected mice)] × 100}.

Statistical analysis

The statistical analyses were performed using the Stat View Ver. 5 software program (SAS Institute Inc., NC, USA). The statistical significance of the findings was calculated using the Mantel-Cox log rank test for survival experiments. The unpaired *t*-test was used for the in vitro assay. *p*-Values of less than 0.05 were considered to be statistically significant. All values are represented as the mean ± standard deviation.

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References

Arnon, T.I., Lev, M., Katz, G., Chernobrov, Y., Porgador, A., Mandelboim, O., 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur. J. Immunol. 31 (9), 2680–2689.

Barber, D.L., Wherry, E.J., Ahmed, R., 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. J. Immunol. 171 (1), 27–31.

Bender, B.S., Croghan, T., Zhang, L., Small Jr., P.A., 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. 175 (4), 1143–1145.

Brigl, M., Bry, L., Kent, S.C., Gumperz, J.E., Brenner, M.B., 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat. Immunol. 4 (12), 1230–1237.

Carnaud, C., Lee, D., Donnars, O., Park, S.H., Beavis, A., Koezuka, Y., Bendelac, A., 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J. Immunol. 163 (9), 4647–4650.

Cerundolo, V., Silk, J.D., Masri, S.H., Salio, M., 2009. Harnessing invariant NKT cells in vaccination strategies. Nat. Rev. Immunol. 9 (1), 28–38.

De Santo, C., Salio, M., Masri, S.H., Lee, L.Y., Dong, T., Speak, A.O., Porubsky, S., Booth, S., Veerapen, N., Besra, G.S., Grone, H.J., Platt, F.M., Zambon, M., Cerundolo, V., 2008. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. J. Clin. Invest. 118 (12), 4036–4048.

Diana, J., Lehuen, A., 2009. NKT cells: friend or foe during viral infections? Eur. J. Immunol. 39 (12), 3283–3291.

Duthie, M.S., Kahn, M., White, M., Kapur, R.P., Kahn, S.J., 2005. Critical proinflammatory and anti-inflammatory functions of different subsets of CD1d-restricted natural killer T cells during *Trypanosoma cruzi* infection. Infect. Immun. 73 (1), 181–192.

Exley, M.A., Bigley, N.J., Cheng, O., Tahir, S.M., Smiley, S.T., Carter, Q.L., Stills, H.F., Grusby, M.J., Koezuka, Y., Taniguchi, M., Balk, S.P., 2001. CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. J. Leukoc. Biol. 69 (5), 713–718.

Gazit, R., Gruda, R., Elboim, M., Arnon, T.I., Katz, G., Achdout, H., Hanna, J., Qimron, U., Landau, G., Greenbaum, E., Zakay-Rones, Z., Porgador, A., Mandelboim, O., 2006. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat. Immunol. 7 (5), 517–523.

Godfrey, D.I., Kronenberg, M., 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. J. Clin. Invest. 114 (10), 1379–1388.

Gonzalez-Aseguinolaza, G., de Oliveira, C., Tomaska, M., Hong, S., Bruna-Romero, O., Nakayama, T., Taniguchi, M., Bendelac, A., Van Kaer, L., Koezuka, Y., Tsuji, M., 2000. alpha -galactosylceramide-activated Valpha 14 natural killer T cells mediate protection against murine malaria. Proc. Natl. Acad. Sci. U. S. A. 97 (15), 8461–8466.

Grubor-Bauk, B., Simmons, A., Mayrhofer, G., Speck, P.G., 2003. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. J. Immunol. 170 (3), 1430–1434.

Grubor-Bauk, B., Arthur, J.L., Mayrhofer, G., 2008. Importance of NKT cells in resistance to herpes simplex virus, fate of virus-infected neurons, and level of latency in mice. J. Virol. 82 (22), 11073–11083.

Gumperz, J.E., Brenner, M.B., 2001. CD1-specific T cells in microbial immunity. Curr. Opin. Immunol. 13 (4), 471–478.

Ho, L.P., Denney, L., Luhn, K., Teoh, D., Clelland, C., McMichael, A.J., 2008. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. Eur. J. Immunol. 38 (7), 1913–1922.

Ishikawa, H., Ochi, H., Chen, M.L., Frenkel, D., Maron, R., Weiner, H.L., 2007. Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody. Diabetes 56 (8), 2103–2109.

Ishikawa, H., Tanaka, K., Maeda, Y., Aiba, Y., Hata, A., Tsuji, N.M., Koga, Y., Matsumoto, T., 2008. Effect of intestinal microbiota on the induction of regulatory CD25+ CD4+ T cells. Clin. Exp. Immunol. 153 (1), 127–135.

Johnson, T.R., Hong, S., Van Kaer, L., Koezuka, Y., Graham, B.S., 2002. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. J. Virol. 76 (9), 4294–4303.

Kakimi, K., Guidotti, L.G., Koezuka, Y., Chisari, F.V., 2000. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J. Exp. Med. 192 (7), 921–930.

Kinjo, Y., Wu, D., Kim, G., Xing, G.W., Poles, M.A., Ho, D.D., Tsuji, M., Kawahara, K., Wong, C.H., Kronenberg, M., 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 434 (7032), 520–525.

Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M.R., Zajonc, D.M., Ben-Menachem, G., Ainge, G.D., Painter, G.F., Khurana, A., Hoebe, K., Behar, S.M., Beutler, B., Wilson, I.A., Tsuji, M., Sellati, T.J., Wong, C.H., Kronenberg, M., 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat. Immunol. 7 (9), 978–986.

Kosaka, A., Wakita, D., Matsubara, N., Togashi, Y., Nishimura, S., Kitamura, H., Nishimura, T., 2007. AsialoGM1+CD8+ central memory-type T cells in unimmunized mice as novel immunomodulator of IFN-gamma-dependent type 1 immunity. Int. Immunol. 19 (3), 249–256.

Kulkarni, R. R., Haeryfar, S. M., and Sharif, S. The invariant NKT cell subset in anti-viral defenses: a dark horse in anti-influenza immunity? J. Leukoc. Biol.

Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T.I., Bushkin, Y., Davis, D.M., Strominger, J.L., Yewdell, J.W., Porgador, A., 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409 (6823), 1055–1060.

Mattner, J., Debord, K.L., Ismail, N., Goff, R.D., Cantu III, C., Zhou, D., Saint-Mezard, P., Wang, V., Gao, Y., Yin, N., Hoebe, K., Schneewind, O., Walker, D., Beutler, B., Teyton, L., Savage, P.B., Bendelac, A., 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature 434 (7032), 525–529.

Mendiratta, S.K., Martin, W.D., Hong, S., Boesteanu, A., Joyce, S., Van Kaer, L., 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity 6 (4), 469–477.

Nagarajan, N.A., Kronenberg, M., 2007. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J. Immunol. 178 (5), 2706–2713.

Nieuwenhuis, E.E., Matsumoto, T., Exley, M., Schleipman, R.A., Glickman, J., Bailey, D.T., Corazza, N., Colgan, S.P., Onderdonk, A.B., Blumberg, R.S., 2002. CD1d-dependent macrophage-mediated clearance of *Pseudomonas aeruginosa* from lung. Nat. Med. 8 (6), 588–593.

Noda, S., Tanaka, K., Sawamura, S., Sasaki, M., Matsumoto, T., Mikami, K., Aiba, Y., Hasegawa, H., Kawabe, N., Koga, Y., 2001. Role of nitric oxide synthase type 2 in acute infection with murine cytomegalovirus. J. Immunol. 166 (5), 3533–3541.

Smiley, S.T., Kaplan, M.H., Grusby, M.J., 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science 275 (5302), 977–979.

Stein-Streilein, J., Guffee, J., 1986. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. J. Immunol. 136 (4), 1435–1441.

Stober, D., Jomantaite, I., Schirmbeck, R., Reimann, J., 2003. NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted CD8+ T cells in vivo. J. Immunol. 170 (5), 2540–2548.

Tupin, E., Kinjo, Y., Kronenberg, M., 2007. The unique role of natural killer T cells in the response to microorganisms. Nat. Rev. Microbiol. 5 (6), 405–417.

Van Dommelen, S.L., Degli-Esposti, M.A., 2004. NKT cells and viral immunity. Immunol. Cell Biol. 82 (3), 332–341.

van Dommelen, S.L., Tabarias, H.A., Smyth, M.J., Degli-Esposti, M.A., 2003. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. J. Virol. 77 (3), 1877–1884.

Wesley, J.D., Tessmer, M.S., Chaukos, D., Brossay, L., 2008. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. PLoS Pathog. 4 (7), e1000106.

Wysocka, M., Bennink, J.R., 1988. Limiting dilution analysis of memory cytotoxic T lymphocytes specific for individual influenza virus gene products. Cell. Immunol. 112 (2), 425–429.